In-vitro antitumor activity of new quaternary phosphonium salts, derivatives of 3-hydroxypyridine

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This work presents the results of in-vitro biological activity studies of three novel anticancer agents, phosphonium salts based on the 3-hydroxypyridine scaffold, including one derivative of 4-deoxypyridoxine. Proliferation and viability of cells treated with these compounds was assessed by the colony formation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Effects of the compounds on apoptosis and cell cycle were studied by flow cytometry using annexin V-FITC/propidium iodide and propidium iodide staining, respectively. The influence of the compounds on mitochondrial membrane potential and intracellular reactive oxygen species was evaluated using tetramethyl rhodamine ethyl and DCFHA staining. Western blot analysis was used to study the changes in the expression of Bcl-xL, Bax, and caspase-3 apoptotic proteins. The treatment of ovarian adenocarcinoma cells OVCAR-4 with the tested compounds inhibited the growth and induced cell cycle arrest in the G1 phase. 3-Hydroxypyridine derivatives induced apoptosis by hyperexpression of Bax and caspase-3, whereas 4-deoxypyridoxine derivative induced cell death partly by reactive oxygen species generation and caspase-3 hyperexpression. These results indicate that the quaternary phosphonium salts studied represent potential therapeutic agents for the treatment of ovarian cancer. ***Anti-Cancer Drugs*** **00:000–000**

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Introduction

Cancer is one of the leading causes of morbidity and mortality worldwide, with ~5.4 million deaths in 2017 (*http://www.worldometers.info/*). Ovarian cancer is the eighth most common cancer among women, and it accounts for about 4% of all cancers in women [1]. According to global estimates, 225 000 new cases are detected each year, and 140 000 individuals die annually from the disease [1]. Subsequently, novel approaches are required for improvement of ovarian cancer therapies.

Phosphonium salts are one of the promising classes of compounds with antitumor activity. Thus, a combination of ampicillin with phosphonium cations forms active ionic liquids that show potent antiproliferative activities against five different human cancer cell lines and low cytotoxicity against two primary cell lines – skin and gingival fibroblasts [2]. A series of triphenylphosphonium salts with remarkable activity against a panel of cancer cell lines as well as in a mouse model of human breast cancer has been reported [3]. Betulinic acid derivatives with one or two triphenylphosphonium moieties show considerable antitumor activities against the tested cancer murine and human tumor cell lines [4]. Gamitrinib modified by triphenylphosphonium cation is a mitochondria-targeted Hsp90 inhibitor that increases cell death in HeLa and MCF-7 cells [5].

Some pyridine derivatives also showed anticancer activity. For example, 2,6-diaryl substituted pyridine showed moderate cytotoxic against different human cancer cell lines [6]. 3-Cyanopyridine compounds with different alkyl or (hetero)aryl groups were found to possess anticancer activity [7]. 2,6-Dibenzylamino-3,5-dicyanopyridines showed potential and promising anticancer activity toward cell lines of nine different types of human cancer [8]. Pyridine derivative LTU-27 (8-methyl-2-(morpholine-4yl)-7-(pyridine-3-methoxy)-4H-1,3-benzoxacine-4-one) was shown to sensitize cancerous cells, specifically lung cancer and colon cancer, to the effects of radiation [9].

In our previous work, different phosphonium salts based on the 3-hydroxypyridine scaffold were synthesized. Some of them possessed pronounced antitumor activity against MCF-7 breast cancer cells [10]. This work presents the results of systematic in-vitro biological activity studies of three leading compounds from this novel interesting chemotype of anticancer agents.

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Methods

Compounds

Phosphonium salts **1–3** studied in this work (Fig. 1) were synthesized according to our recently reported approaches [10]. The reaction of 3-hydroxypyridine with paraformaldehyde in alkaline medium led to 2-monohydroxymethyl and 2,6-*bis*-hydroxymethyl derivatives, which were converted into the corresponding chlorides by reaction with thionyl chloride. At the last step, the chlorides were treated with an excess of *tris*(*p*-tolyl) phosphine to yield the target phosphonium salts **1** and **2**. Compound **3** was synthesized from 4-deoxypyridoxine, which was treated with thionyl chloride to yield the corresponding 5-chloromethyl derivative. Acylation of the aromatic hydroxy group with acetyl chloride, followed by reaction of the resulting intermediate with *tris*(*p*-tolyl) phosphine yielded phosphonium salt **3**. The developed syntheses are straightforward and well reproducible.

Cell culture

Human ovarian adenocarcinoma cells OVCAR-4 (GSM136312) and primary human foreskin fibroblast cells (HSF) isolated from the skin explant according to conventional protocol [11] were cultured in α-MEM (PanEko, Moscow, Russia) supplemented with 10% fetal bovine serum (PAA; PAA Laboratories, Morningside QLD, Australia), L-glutamine, and 1% penicillin–streptomycin at 37°C in a 5% humidified CO_2 atmosphere in air. Cells were removed from the culture substrate by treatment with trypsin-EDTA with subsequent inactivation of trypsin by adding α-MEM-containing serum. Cell suspensions were precipitated by centrifugation at 500*g* and pellet was resuspended in PBS. Cell viability and density were measured in a Neubauer chamber using a 0.4% solution of Trypan blue. Suspensions with the amount of viable cells no less than 90% were used in the experiments.

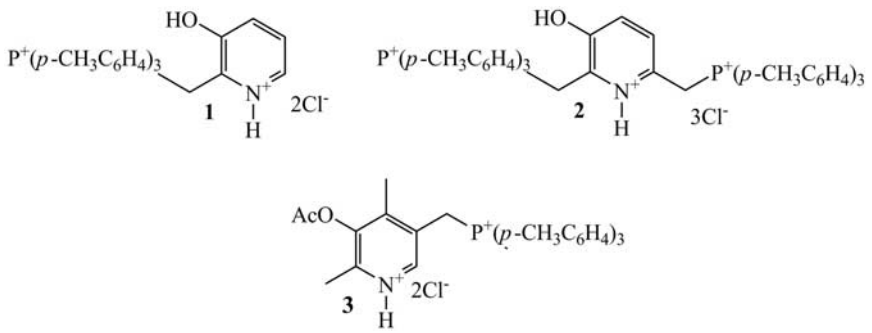
Colony formation assay

Cells (1000 cells/well) were cultured in 980 µl of the culture medium according to the culture conditions in a six-well plate for 24 h. The medium was then changed to 980 µl of a new culture medium, and 20 µl of compounds were added and incubated under standard conditions for 7 days. The concentration of stock solutions was 300 µmol/l. After incubation, the colonies were washed three times with a cold solution of PBS and fixed in a fixing buffer (glacial acetic acid, methanol, distilled water in a 10 : 10 : 80 ratio). The colonies were then stained with a 0.4% alcoholic solution of crystal violet. The results were presented as the percentage of control values (untreated cells).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-assay

The cytotoxicity of compounds on OVCAR-4 culture was assessed using a proliferative 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT) (Promega, Madison, Wisconsin, USA). Cells (1000 cells/well) were cultured in 90 µl of the culture medium according to the culture under standard conditions in a 96-well plate for 24 h. Then, 10 µl of the test preparations were added and incubated under standard conditions for 6, 24, 48, and 72 h. The concentrations of stock solutions were 300 µmol/l for **1** and **2**; 1800 µmol/l for **3**; and 100 µmol/l for doxorubicin. The medium with the tested samples was replaced with a nutrient medium (80 µl), and then 20 µl of MTT reagent (5 mg/l) was added, the mixture was incubated for 3.5 h, the medium was removed, and 100 µl of DMSO was added. After 10 min, the optical density of the cell solutions was measured at 555 nm (reference wavelength 650 nm) on a TECAN plate reader (Switzerland). Data were presented as percentage of MTT reduction compared with the control cells. For the tested drugs, a dose–response curve was obtained, and IC_{50} values ($P < 0.05$) were calculated.

Fig. 1



Structures of phosphonium salts based on 3-hydroxypyridine and 4-deoxypyridoxine: 3-hydroxy-2-[tris(*p*-tolyl)phosphoniomethyl] pyridinium dichloride **(1)**, 3-hydroxy-2,6-*bis*-[tris(*p*-tolyl)phosphoniomethyl] pyridinium dichloride **(2)**, 3-acetoxy-2,4-dimethyl-5-[tris(*p*-tolyl)phosphoniomethyl] pyridinium chloride **(3)**.

Apoptotic assay

OVCAR-4 cells were seeded in 25 cm^2 flasks till nearly 80% confluence. Media were changed to media containing compound **1**, **2**, or **3** in a concentration equal to IC_{50}. After a 48-h incubation, the cells were harvested by trypsinization to detach the cells without affecting the integrity of the cell membrane. The cells were washed in PBS and 1×10^5 cells were collected by centrifugation. Then, cells were resuspended in 300 µl of 1× binding buffer, and 1 µl of annexin V-FITC and 1 µl of propidium iodide (PI) (50 µg/ml) were added. The cells were incubated at room temperature for 30 min in the dark. The fluorescence intensity of annexin V-FITC was determined by the FL1-H channel and PI by the FL3-H channel on the Guava easyCyte 8HT Flow Cytometer (Millipore, Millipore Corporation, Burlington, Massachusetts, USA).

Cell cycle

Assessment of cell cycle distribution and cell proliferation is important to study cell growth differentiation and apoptosis. OVCAR-4 cells were seeded in 25 cm^2 flasks till nearly 80% confluence. Media were changed to media containing compound **1**, **2**, or **3** in $IC_{50.}$ After a 48-h incubation, the cells were harvested by trypsinization to detach the cells without affecting the integrity of the cell membrane. The cells were washed in PBS, pelleted by centrifugation at 1500 rpm, and fixed in ice-cold 70% ethanol while vortexing. Cells were fixed for 4 h at 4°C and washed in PBS twice by centrifugation at 1500 rpm. Then, 50 µl of a 10 mg/ml stock of RNase, 500 µg/ml PI, and 10% Triton-X10 were added and incubated at room temperature for 30 min in the dark. The PI fluorescence intensity in the cells was measured using a Guava easyCyte 8HT Flow Cytometer at excitation and emission wavelengths of 536 and 617 nm, respectively, using an Fl2-H filter.

Determination of mitochondrial membrane potential and reactive oxygen species

A fluorometric assay using intracellular dye tetramethyl rhodamine ethyl (TMRE) for the measurement of mitochondrial membrane potential (MMP) and intracellular oxidation of 2,7-dichlorohydrofluorescein diacetate (DCFH-DA) for the measurement of reactive oxygen species (ROS) in cells was performed. OVCAR-4 cells were seeded in T-25 flasks at a density of 5×10^6 cells/flask and were incubated at 37°C under a humidified atmosphere containing 5% CO_2 for 24 h before treatment. The cells were incubated in full α-MEM media containing compounds in a concentration equal to IC_{50}. As a negative control sample, we used Milli-Q water, which was obtained using a water filtration station (Millipore Corporation, Burlington, Massachusetts, USA). After 48 h, the medium was removed and washed with PBS. Then, cells were harvested with trypsin-EDTA with subsequent inactivation of trypsin by adding α-MEM-containing serum. Cell suspensions were precipitated by centrifugation at 500*g* and the pellet was resuspended in PBS to a final density of 2×10^6 cells/ml. To 100 µl of cell suspensions, 1 µl TMRE solution (17 µmol/l) or 1 µl DCFH-DA solution (200 µg/ml) was added to each well, followed by 20 min of incubation at 20°C. Subsequently, the medium was removed and washed twice with PBS. Then, the fluorescence of TMRE (ex. 488 nm, em. 575 nm) or dichlorofluorescein, which is the oxidized product of DCFH-DA (ex. 485 nm, em. 530 nm), was measured using the TECAN plate reader. Data were expressed as the percentage of the ROS level in the control group.

Immunoblot analysis and source of antibodies

The cells were harvested, washed in PBS, and lysed in ice-cold RIPA buffer (50 mmol/l Tris pH 7.4, 150 mmol/l NaCI, 1 mmol/l EDTA, 0.25% sodium deoxycholate, 1.0% NP-40, and protease inhibitors) at 4°C for 20 min. Cell lysates were normalized (Bradford assay) and equal protein loading was confirmed with Ponceau S staining. Total protein (20–30 µg) was resolved by denaturing 12% SDS-PAGE gel electrophoresis before transfer to a nitrocellulose membrane (Inverclyde Biologicals, Bellshill, Lanarkshire, UK) and probed with antibody overnight at 4°C. Membranes were incubated with the corresponding secondary HRP antibodies for 1 h at room temperature before a signal was detected using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, Illinois, USA) and the Bio-Rad ChemiDOC system. The following antibodies were used in this study: monoclonal antibodies against Bax (Abcam, Cambridge, UK), Bcl-2 (Abcam), caspase-3 (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), and anti-β-actin (Abcam). The secondary antibodies included HRP-conjugated anti-rabbit IgG (Abcam).

Results

Colony formation assay

The ability of the OVCAR-4 cells to maintain their clonogenic capacity and form colonies after treatment was evaluated using a clonogenic assay. Compounds **1**, **2**, **3** and DOX induced a dose-dependent growth inhibition after 7 days of exposure. The calculated IC_{50} values ranged from 0.09 to 2.1 µmol/l (Table 1). Doxorubicin and compound **3** had slightly higher antiproliferative activity compared with **1** and **2**.

Table 1 Effect of compounds on colony-forming capacity in OVCAR-4 cells by a clonogenic assay

Compounds	IC_{50} (µmol/l)
1	2.06 ± 0.13
2	0.36 ± 0.08
3	0.10 ± 0.00
DOX	0.09 ± 0.00

The data represent the mean ± SEM of three independent experiments.

Cytotoxicity in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-assay

The cytotoxic activity of compounds against OVCAR-4 and HSF cell lines was examined using the MTT assay. All compounds induced time-dependent and dose-dependent growth inhibition (Table 2). After 72-h exposure, the calculated IC_{50} values ranged from 0.91 to 16 μmol/l for OVCAR-4 cells and from 0.76 to 65 μmol/l for HSF. Compounds **1** and **3** have better selectivity indexes (SIs, 5.1 and 4.1, respectively) compared with doxorubicin and **2**. The IC_{50} values obtained after 72-h exposure were used for subsequent experiments.

Apoptosis detection by flow cytometry

The effect of compounds on apoptosis was evaluated using the PI and annexin V-FITC biparametric cytofluorimetric analysis. After treatment for 48 h with the tested compounds in concentrations equal to IC_{50}, OVCAR-4 cells were stained with PI and annexin V-FITC, and then analyzed using flow cytometry. The results are presented in Table 3. No early or delayed manifestations of apoptosis were found in cells treated with compound **3**, but the treatment of cells with compounds **1** and **2** for 48 h resulted in increased percentage of the late-stage apoptotic cells by ~1.9 and 3 times, respectively.

Inhibition of cell growth induced by the studied quaternary phosphonium salts has been associated with a significant level of cellular death, but this assay does not distinguish between cells that have undergone apoptotic death versus those that have died as a result of a necrotic pathway as in both cases, the dead cells are stained with both FITC annexin V and PI.

Cell cycle analysis by flow cytometry

It was shown that all compounds reduce the number of viable OVCAR-4 cells in a dose-dependent manner. To determine whether the studied compounds regulate cell cycle progression in OVCAR-4 cells, the cells were treated for 48 h with concentrations equal to IC_{50} and then DNA was stained with PI, followed by FACS analysis (Fig. 2).

The treatment of cells with **1**, **2**, and **3** for 48 h resulted in increased percentage of cells in the G1 phase, with a concomitant reduction in cell numbers in the S phase. After the treatment of OVCAR-4 cells with compounds **1**, **2**, and **3**, the cell population in the G0/G1 phase was 67, 69, and 70%, respectively, compared with 49% cell population in the G0/G1 phase in the control group. At the same time, the cell population in the S phase was 25, 23, and 21% after the treatment with compounds **1**, **2**, and **3**, respectively, compared with 42% cell population in the S phase in the control group. However, the differences between the experimental groups in the G2/M phase were not statistically significant.

Mitochondrial membrane potential (ΔΨ)

The effect of compounds on the MMP was studied using TMRE that was accumulated within the mitochondria in inverse proportion to MMP according to the Nernst equation. The polarized mitochondria are capable of accumulating more cationic dye than depolarized ones. Figure 3 shows that the mitochondrial membrane potential of OVCAR-4 cells was decreased after treatment with compound **2**. The intracellular fluorescent intensity of cells was 8100 relative fluorescence units (RFU), lower than that of the control group (10 000 RFU). The results indicated that compound **2** induced mitochondrial dysfunction and apoptosis as shown in Fig. 4 and Table 3. At the same time, compounds **1** and **3** did not alter the mitochondrial membrane potential of OVCAR-4 cells.

ROS generation in OVCAR-4 cells

To evaluate whether the tested compounds could increase ROS levels, OVCAR-4 cells were treated with **1**, **2**, and **3** in concentrations equal to IC_{50} for 48 h. As shown in Fig. 5, **1** and **3** boosted ROS generation. After the treatment of OVCAR-4 cells with compounds **1** and **3**, the intracellular dichlorofluorescein fluorescence intensity was 9200 and 11 000 RFU, respectively, compared with 8000 RFU in the

Table 2 Cytotoxicity of compounds in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-assay

		Cytotoxicity, IC_{50} (μmol/l)			
	Compounds	6 h	24 h	48 h	72 h
OVCAR	**1**	>30	4.4±1.0	1.1±0.04	1.9±0.02
	2	>30	>30	>30	8.4±0.6
	3	>180	>180	49±15	16±6
	DOX	>10	3.9±0.5	3.1±0.5	0.91±0.1
HSF	**1**	>30	>30	>30	9.6±0.5
	2	>30	>30	>30	19±8
	3	>180	>180	>180	65±8
	DOX	>10	>10	>10	0.76±0.05
Selectivity index $IC_{50\ HSF}/IC_{50\ OVCAR-4}$	**1**	–	–	–	5.1
	2	–	–	–	2.3
	3	–	–	–	4.1
	DOX	–	–	–	0.9

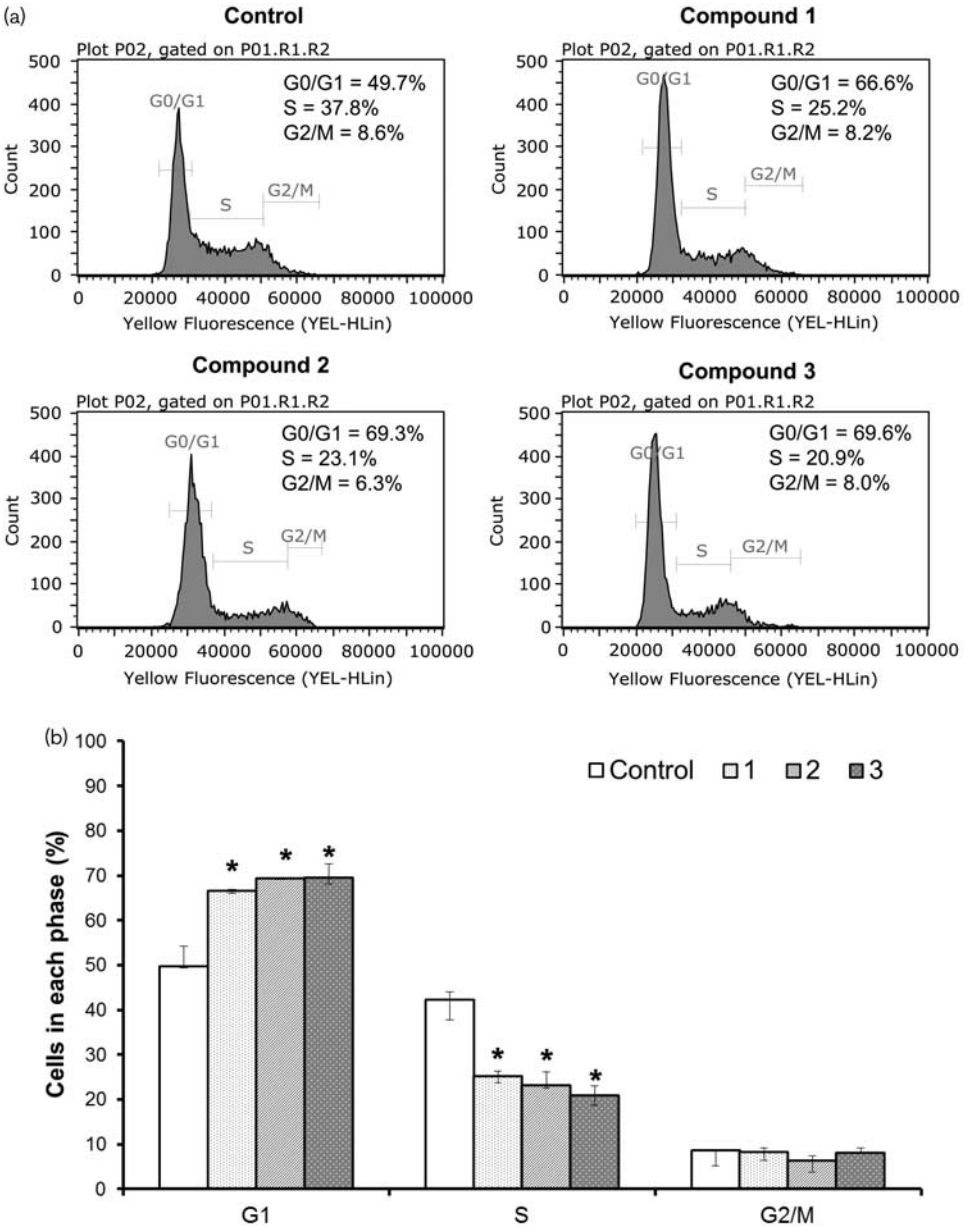
The data represent the mean±SEM of three independent experiments.
HSF, human skin fibroblast.

Table 3 Effect of compounds on apoptosis of OVCAR-4 cells in concentrations equal to IC_{50} (1–1.9 μmol/l, 2–8.5 μmol/l, 3–9 μmol/l)

	%Gated			
	1	**2**	**3**	Control
Live (LL)	77.6±1.3	81.8±1.0	84.5±0.5	92.1±0.7
Apoptotic (LR)	0.02±0.02	0.01±0.0	0.03±0.02	0.02±0.02
Apoptotic/dead (UR)	0.28±0.08*	0.44±0.06*	0.13±0.05	0.15±0.02
Dead (UL)	22.1±1.4*	17.9±1.1*	15.3±0.5*	7.8±0.7

Apoptosis was measured using flow cytometric analysis of annexin V binding 48 h after treatment. The presented values are calculated from the flow cytometry plots (Fig. 3). LL, lower left; LR, lower right.; UL, upper left; UR, upper right.
*Values indicate $P<0.05$.

Fig. 2



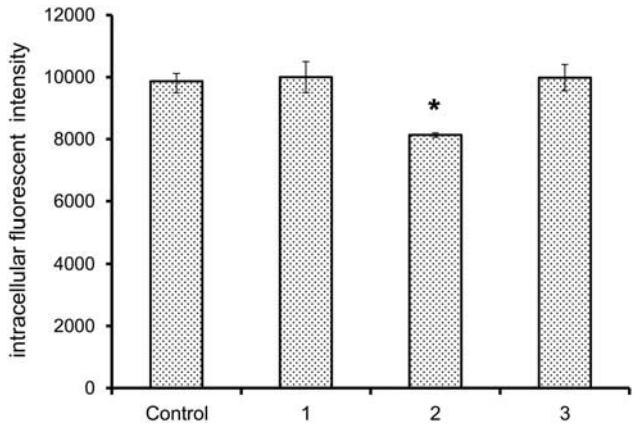
Effect of compounds **1–3** on OVCAR-4 cell cycle arrest. Cells were treated with compounds and harvested after 48 h. Then, they were fixed with ethanol, stained with propidium iodide, and analyzed by flow cytometry. (a) Cell distribution histograms. (b) Percentage of cells in the G0/G1, S, and G2/M phases (data are presented as mean ± SD of three independent experiments). *Values indicate $P < 0.05$.

control group. These results suggested that **1** and **3** induced cellular toxicity in OVCAR-4 cells, at least partly by ROS generation. Interestingly, under the same experimental conditions, compound **2** did not increase the intracellular ROS concentration.

Expression of apoptosis-related proteins

In the next experiment, we attempted to elucidate the role of Bcl-xL, Bax, and caspase-3 in the antitumor activity of the tested compounds. OVCAR-4 cells were treated with the compounds in concentrations equal to IC_{50} for 48 h; as a control, untreated cells were used. Lysates of the treated and untreated cells were prepared and processed for western blot analysis to study the changes in the levels of apoptosis-related proteins. The level of Bcl-xL protein did not change, whereas the level of Bax protein increased after the treatment with compounds **1** and **2**. The relative ratio of Bax to Bcl-2 was increased. Furthermore, the expression of caspase-3 protein was significantly increased with all compounds (Fig. 6).

Fig. 3



Effect of compounds **1–3** on the mitochondrial membrane potential (Dψm). The OVCAR-4 cells treated were stained with TMRE and the intensity of tetramethyl rhodamine ethyl fluorescence in each sample was analyzed using a TECAN plate reader. Data are presented as mean ± SD (relative fluorescence units, RFU) of three independent experiments. *Values indicate $P < 0.05$.

The obtained results suggest that compounds **1** and **2** induce apoptosis by hyperexpression of Bax and caspase-3, but compound **3** probably acts by alternative mechanisms associated with triggering of cell death by ROS generation and caspase-3 hyperexpression.

Discussion

Phosphonium salts show great promise for the diagnosis and treatment of neoplasms [3]. Several phosphonium salts, because of their ability to disrupt mitochondrial structure and alter cellular lipid content, have shown antiproliferative activity in a panel of cancer cell lines and in an in-vivo model of ovarian cancer [3]. The diagnostic imaging studies [12] have elucidated two key features of phosphonium salts: (a) they are capable of preferentially accumulating within tumor cells and (b) the phosphonium cation itself is not cytotoxic.

In this study, we assessed the effects of three quaternary phosphonium salts on the basis of 3-hydroxypyridine and 4-deoxypyridoxine (Fig. 1) on OVCAR-4 ovarian cancer cells. Treatment of these cells with *tris*(*p*-tolyl)phosphine **1** and *bis-tris*(*p*-tolyl)phosphine **2** derivatives of 3-hydroxypyridine, and acetoxy-dimethyl-*tris*(*p*-tolyl)phosphoniomethyl derivative **3** of 4-hydroxypyridine was found to inhibit the growth and to induce cell cycle arrest in the G1 phase. According to the results of the clonogenic assay, compound **3** had the highest antiproliferative activity, which was comparable to that of the reference drug doxorubicine. Introduction of an additional phosphonium fragment resulted in increased cytotoxicity. Thus, *bis*-phosphonium compound **2** (IC_{50} 0.36 ± 0.08 μmol/l) was 5.7-fold more cytotoxic than monophosphonium compound **1** (IC_{50} 2.06 ± 0.13 μmol/l).

In comparison with the clonogenic test, the MTT assay enables the assessment of the metabolic cellular activity. It is known that the duration of the OVCAR-4 cell division cycle is 43 h on average. For this reason, in the MTT cytotoxicity assay, incubation with the tested compounds has been performed for 72 h, during which time the dose and time kinetics of the cell survival-modulating action of the tested compounds were measured (Table 2). After 72-h exposure, doxorubicin showed the highest antitumor activity, but the lowest SI, which may explain its expressed side effects. At the same time, all the studied phosphonium salts had a relatively good SI (from 2.3 to 5.1). Interestingly, there were no cytotoxic actions of the studied compounds **2** and **3** before 72 h, which indicates their indirect action by triggering the apoptotic pathways.

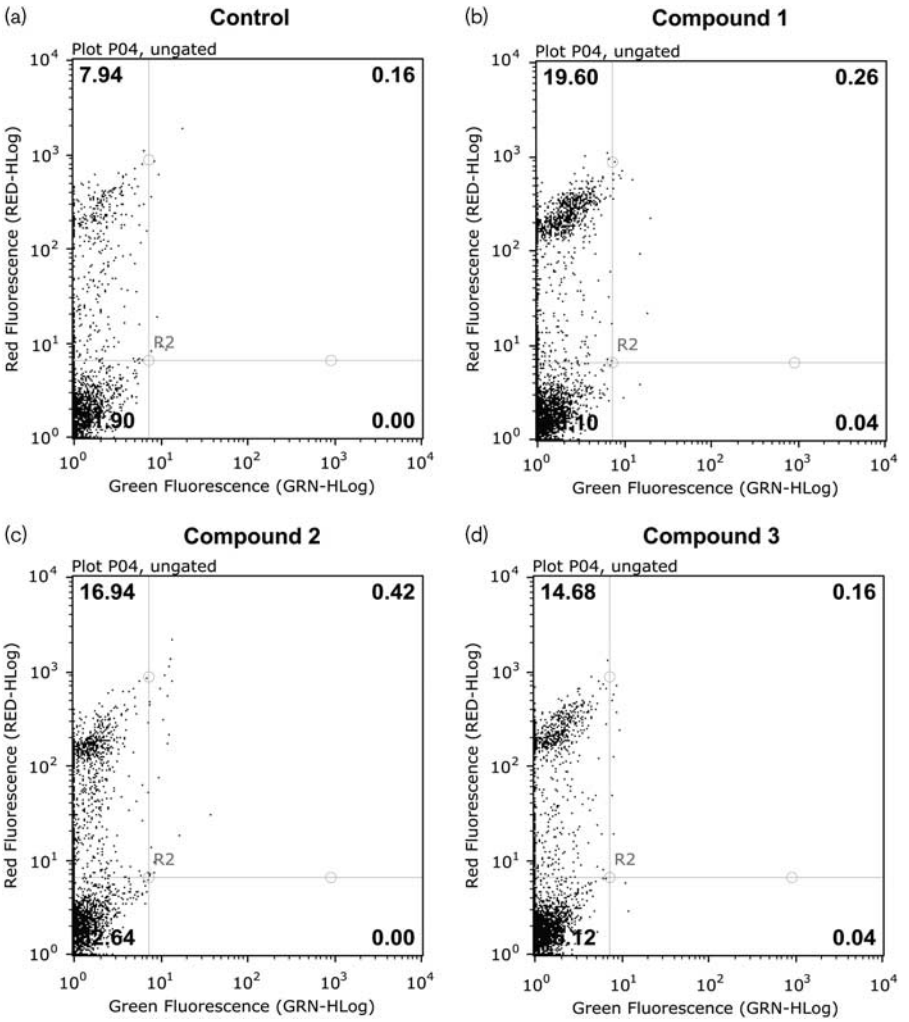
It is accepted widely that the charged molecules are unable to move freely across the cellular membranes without the aid of active transport systems. The distribution of positive charge across the large lipophilic surface of the phosphonium cation significantly lowers the activation energy, thus facilitating passage through the membranes [3]. The positive charge also leads to the accumulation of phosphonium salts in mitochondria because of their negative membrane potential. Given these important features of the triphenylphosphonium cation, it has been used for the targeted mitochondrial delivery of spin traps, fluorescent dyes, and antioxidants. It is important to note that many solid tumors have a more negative mitochondrial membrane potential compared with normal cells [13], and this feature can be used for selective delivery of the phosphonium-modified agents to tumor cells.

At the same time, one should not exclude the possibility of direct effects of the phosphonium-containing compounds on the activity of both mitochondrial reductases, which catalyze the reduction of tetrazolium salts to colored formazan, and on direct transport of the MTT reagent. Therefore, it may be suggested that the affinity of phosphonium compounds to mitochondria positively correlates with the observed IC_{50} values. Thus, compound **3**, which shows the highest activity in the clonogenic test, shows the minimal cytotoxicity in the MTT test; the opposite dependences are observed for compound **1**.

The subsequent evaluation of biological activity was based on IC_{50} values obtained in the MTT experiment (Table 2). Analysis of action of compounds **1–3** on the cell cycle showed that all the tested compounds induced a decreased percentage of cells in the S phase and a proportionally increased percentage of cells in the G1 phase, which indicates the cell cycle arrest in the G1 phase.

One of the key strategies for anticancer drug development is to induce apoptotic events in cancer cells while minimizing the negative effects in normal cells [14,15]. In this work, we have shown that a proportion of cells are killed after incubation with the tested compounds for 48 h, as evidenced by increased percentage of cells

Fig. 4

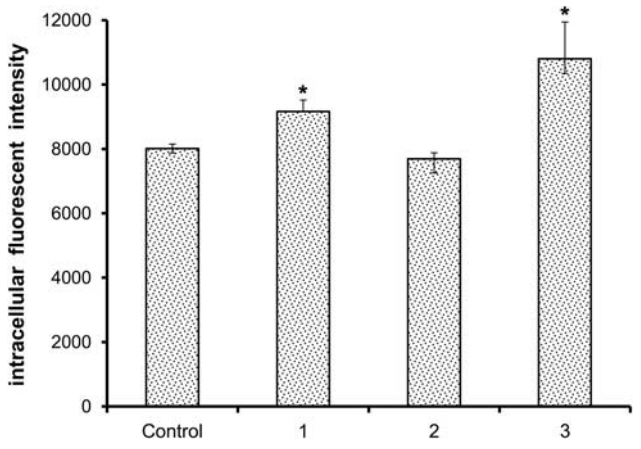


Flow cytometry analysis of the apoptotic effects of compounds **1–3** on OVCAR-4 cells. The scatter dot plots indicating the percentage of live [annexin V negative, propidium iodide (PI) negative], early (annexin V positive, PI negative), and late (annexin V positive, PI positive) apoptotic cells after treatment with medium (control) (a), compound **1** (b), compound **2** (c), and compound **3** (d) for 48 h using annexin V and PI assay.

stained positively with FITC annexin V and PI. It should be noted that the cell death caused by compound **3** is because of an increase in the intracellular level of ROS and also because of overexpression of caspase-3. Activation of caspase-3 enzymes is a marker of apoptosis that can be used in cellular assays to quantify the efficacy of activators and inhibitors of this 'death cascade'. Caspase-3 enzymes are activated in apoptotic cells both by extrinsic (death ligand) and by intrinsic (mitochondrial) pathways. In view of the absence of the influence of compound **3** on the mitochondrial potential and the expression of Bax and Bcl-xL proteins, it can be assumed that this agent induces the externally mediated caspase pathway of apoptosis because of an elevated ROS level.

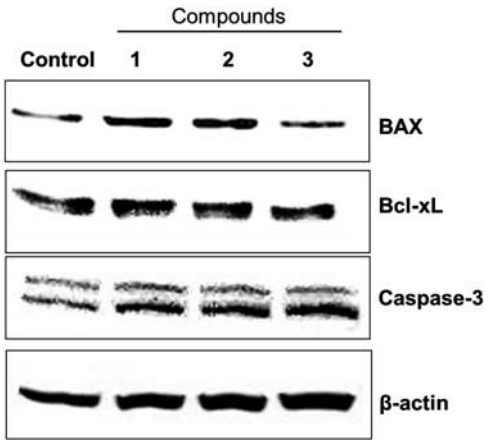
It was reported that the extrinsic pathway can activate the intrinsic pathway by triggering a mitochondrial apoptosis-induced cascade of intracellular signals involving the Bcl-2 protein family. The latter includes both antiapoptotic (e.g. Bcl-xL, Bcl-2) and proapoptotic (e.g. Bax, Bid, tBid, Bad, Bcl-xs) proteins [16]. We have found that compounds **1** and **2** increase the expression of the proapoptotic Bax protein and the main marker of apoptosis, caspase-3. These compounds, in addition to cell death, cause an increase in the percentage of cells in the late stages of apoptosis. Nevertheless, there are differences in the hypothetical mechanism of action of compounds **1** and **2**. Thus, compound **2** has a *bis*-phosphonium fragment in its structure, which reduces the negative membrane mitochondrial potential. It has been shown that the decrease of MMP leads to structural changes in the mitochondria, such as matrix condensation and cristal unfolding [16], which result in the redistribution of cytochrome c from the cristae to the

Fig. 5



Effect of compounds **1**–**3** on ROS concentration. The treated OVCAR-4 cells were stained with DCFH-DA, and the intensity of DCF fluorescence in each sample was analyzed using a TECAN plate reader. Data are presented as mean ± SD (relative fluorescence units, RFU) of three independent experiments. *Values indicate $P < 0.05$.

Fig. 6



Effect of compounds **1**–**3** (IC_{50}) on caspase-3, Bax, and Bcl-xL protein expression in the treated and control groups. Cells were incubated with compounds **1**, **2,** and **3** in concentrations equal to IC_{50} or with media (control) for 48 h. Lysates containing 20 μg of the protein were subjected to SDS-PAGE, transferred to nitrocellulose, immunoblotted with anti-caspase-3, anti-Bcl-xL, and anti-Bax, and detected using the ECL Amersham kit. β-Actin was used as a lane loading control.

intermembrane space, making it more susceptible to release [16]. At the same time, the monophosphonium fragment in compound **1** does not lead to a decrease in the membrane mitochondrial potential, and this agent exerts its action mainly by increasing the level of ROS.

Conclusion

In this work, we have studied the mechanisms of anticancer activity of three monophosphonium and *bis*-phosphonium derivatives of 3-hydroxypydoxine. Despite obvious structural similarity, there are some differences in the mechanisms of anticancer action of compounds **1**–**3**. Specifically, the monophosphonium fragment in compound **1** does not lead to a decrease in the membrane mitochondrial potential, and this agent exerts its action mainly by increasing the level of ROS. Our experimental results also show that compound **1** induces apoptosis by hyperexpression of Bax and caspase-3. Compound **2** has a *bis*-phosphonium fragment in its structure, which reduces the negative membrane mitochondrial potential, thus leading to the release of cytochrome C to the intermembrane space and initiation of apoptosis by hyperexpression of Bax and caspase-3. Compound **3** has the highest antiproliferative activity, at least partly by ROS generation. It also induces apoptosis by hyperexpression of caspase-3, rather than proapoptotic proteins. It should be stressed that the mechanisms described are just hypothetical, and further studies are required to validate these. In general, these results suggest that new quaternary phosphonium salts based on 3-hydroxypyridine derivatives represent potential therapeutic agents for the treatment of ovarian cancer.

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Conflicts of interest

There are no conflicts of interest.

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